SUMMARY

ISOLATION AND DIVERSITY OF HALOALKALIPHILIC BACTERIA

- Thirty four different haloalkaliphilic bacteria were isolated from salt-enriched soil collected from salt panes located in Okha (Latitude 22.20 N, Longitude 70.05 E), Gujarat, India. Isolates were initially diversified based on their enrichment conditions followed by the studies on colony characteristics, gram and metabolic reactions, cell morphology, antibiotic profiles, enzyme secretion and protease amplification profile.

- Based on 16S rRNA gene homology, three potential isolates, O.M.$A_1$8, O.M.$E_1$2, O.M.$C_2$8 were related to their nearest homologus and the sequences were deposited in NCBI as *Oceanobacillus iheyensis* O.M.$A_1$8, *Haloalkaliphilic bacterium* O.M.$E_1$2 and *Oceanobacillus oncorhynchi* O.M.$C_2$8. The two potential isolates, O.M.$A_1$8 and O.M.$E_1$2 were also analyzed by FAME confirming their status as genus *Bacillus.*

- Along with classical and molecular parameters, proteolytic activity was considered as one of the parameter for judging the diversity of haloalkaliphilic bacteria. Isolates secreted alkaline proteases in the broad range of NaCl (0-20%, w/v) and pH (8-11) and were further diversified on the basis of their optimum pH and salt. Alkaline protease genes were amplified by using sets of primers. Diversity was evident in terms of product size and concentration of amplicons. Size of the protease gene products varied form 0.5 to 1.2 kb.

- The antibiogram profile was quite useful in generating the microbial diversity profile among the isolates. Overall, isolates were more resistant against Tobramycine and highly sensitive towards Oleandomycin, Cephaloridine, Norfloxacin, Cephradin, Erythomycin, and Cefuroxime. The biochemical and metabolic reactions revealed the production of catalase and oxidase by all isolates. Isolates were diversified on the basis of sugar utilization and none

produced gas in Durham's tube. Only few isolates from O.M.6.2 and O.M.6.5 utilized all the sugars.

PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASES

- The enzyme characteristics reflected that although O.M.$A_1$8 and O.M.$E_1$2 strains were from the same site they reflected quite distinct traits.

- The enzymes were purified to their homogeneity by single step on phenyl sepharose 6 FF affinity column. The purification was achieved with specific activities of 16945 and 24600 U/mg for O.M.$A_1$8 and O.M.$E_1$2 proteases. The apparent molecular mass of the proteases were estimated 35 and 29 kDa for O.M.$A_1$8 and O.M.$E_1$2, respectively. The effect of NaCl was assessed on enzyme activity and thermostability. NaCl enhanced the enzyme activity of purified enzyme, while activity declined in crude and partial purified stage of the enzyme. For both; O.M.$A_1$8 and O.M.$E_1$2 strains, there was shift in temperature profile with NaCl. Enzyme preparations maintained their activities and stability in wide range of pH; 8-11, with maximum activity at 10.

- One of the foremost points of the study was related to the effect of temperature on enzyme catalysis. The O.M.$A_1$8 enzyme had broader range of temperature for catalysis (37-90^{o}C), while that for O.M.$E_1$2 was over a relatively narrower range (37-70^{o}C). The high activity at 90°C for O.M.$A_1$8, would be among the few reports where mesophilic organisms catalyze reaction at elevated temperature. This feature holds novelty from diversity view point as well; since there may be only limited reports where such a unique and contrasting characteristic features are observed from the same site of isolation.

- The partially purified and purified proteases from O.M.$A_1$8 and O.M.$E_1$2 were subjected to urea denaturation. Partially purified enzyme from O.M.$A_1$8 was exceptionally resistant against urea denaturation at 90^{o}C after 48 hours with 8M urea. The O.M.$E_1$2 enzyme, however, was relatively less resistant to urea. The effect of NaCl was assessed on urea denaturation; the findings revealed

that there was no significant change in the denaturation profile of crude, partially purified and dialyzed enzymes. Crude and partially purified enzymes were quite stable in various commercial detergents and oxidizing and reducing agents.

- On the basis of phylogenetic prediction, the next most homologus sequences for all the proteases sequences were found with the reported extracellular protease sequences. The characteristic features; i.e. G+C content, P_I, aliphatic index, hydrophobicity resembled to known haloalkaline proteases.

- The significance of the work relates to the fact that while alkaline proteases are extensively studied, only few haloalkaliphilic bacteria have been explored towards this end. Despite the fact that the saline habitat in the study possessed significant bacterial diversity, it remains unexplored in terms of it characterization, biocatalytic potential, enzymatic characteristics, enzyme structure-function analysis and phylogenentic status.

- Further, some of the novel features of the enzymes, such as stability over the wide range of pH and salt, catalysis and thermostability of enzyme at higher temperatures make them attractive candidates for future studies. The results are also important from the diversity viewpoint as although, both the strains are from the same site, they displayed distinct features of growth, protease secretion and enzymatic characteristics, highlighting their ecological significance.

CLONING, SEQUENCING, OVER EXPRESSION AND CHARACTERIZATION OF SERINE ALKALINE PROTEASES

- Cloning of alkaline protease genes from the genomic DNA of O.M.$A_1$8 and O.M.$E_1$2 was carried out in an over-expression vector, pET21a^+ (Novagen). Vector construct of O.M.$A_1$8 and O.M.$E_1$2 were transformed in *Escherichia coli* strain $BL_{21.}$ The positive clones exhibiting resistance against ampicillin (30µg/ml) were selected for plasmid isolation.

- With respect to over-expression of protease genes from both isolates, the optimum production of recombinant proteases was at 27^{o}C and 1mM IPTG.

- The molecular weights of both proteases were estimated ~30 kDa, which were quite comparable to the enzymes produced in native haloalkaliphilic organisms.

- Over-expressed proteases were purified to the homogeneity by single step purification using nickel column exploiting His-tag property of pET21a^{+}. High level of purified enzymes obtained as evident from the specific activity and yield.

- Enzymes were characterized for physico-chemical properties, as for their native counterparts. In general, the recombinant enzymes were more sensitive towards NaCl, temperature and chemical denaturant compared to the native O.M.A$_1$8 and O.M.E$_1$2 proteases.

- Molecular characterization of recombinant enzymes; distribution of amino acids, 3D structure analysis, protein secondary structure analysis and hydropathy analysis were carried out. The presence of serine residues in active site confirmed the serine nature of the enzyme. Further, stability of enzyme structure was confirmed by *in-silico* analysis.

METAGENOMICS: ISOLATION OF ENVIRONMENTAL DNA AND CAPTURING ALKALINE PROTEASES GENES

- Several protocols were attempted and optimized for the isolation of metagenomics DNA from O.M.6.2 and O.M.6.5 the saline sites used for the isolation and enrichment of bacterial isolates. DNA extraction methods were evaluated in terms of DNA purity, yield and humic acid content. Diversity based analysis and amenability for molecular biology work was assessed by 16S rRNA amplicons. Followed by amplification profile, metagenomics nature of amplicons was elucidated from DGGE-molecular fingerprinting technique. Beside, the source also provided a huge and comprehensive platform for capturing novel alkaline protease gene sequences. Successful

cloning and expression of alkaline protease from metagenomic derived amplicons revealed interesting features.

- Physico-chemical and molecular characterization of metagenomic protease confirmed that some characteristic properties of un-cultivable clone were quite similar to recombinant clones earlier discussed. However, the enzyme was found to be more sensitive. These findings were further enriched by protein structure and function predictions.

ORGANIC SOLVENTS TOLERANCE OF NATIVE, RECOMBINANT AND METAGENOMIC DERIVED PROTEASES

- Native alkaline proteases exhibited resistance towards organic solvents as compared to recombinant enzymes. While, metagenomics derived enzyme displayed minimum resistance against solvents.

- Similar trends were also reflected with reference to enzyme stability. The results on organic solvents holds novelty as similar results exhibiting comparative view on varied enzyme preparations are not studied extensively.

- Catalysis in the presence of solvents was further studied in combination of salt, pH and temperatures.

- To get more insight into phylogenentic relatedness, the protease sequences of O.M.$A_1$8, O.M.$E_1$2 and metagenome clone O.M.6.2 were aligned with known protease sequences available in the database. The enzyme sequences were quite diverse in its sequence similarity; they were more closely related to enzyme sequences of other halophilic organisms as compared to enzymes studied in the present study.

- The present study highlighted the properties and capabilities of the haloalkaliphilic bacteria and their enzymes. The properties of the O.M.$A_1$8 and O.M.$E_1$2 native enzymes and its recombinant counterparts displayed unique features with its ability to function under extreme conditions. Besides, the biotechnological potentials, extensive studies on diversity, physiology and

metabolic reactions would further be supportive to understand the bioenergetics and adaptation strategies of these organisms as they are subjected to different environmental stresses. Moreover, the wide occurrence of the alkaline protease and their properties among cultivable isolates as well by soil derived clone clearly indicated that they could be used as marker for studying bacterial heterogeneity and population dynamics of unexplored habitats. The result on catalytic efficiency in the presence of organic solvents significantly addressed that they can be employed in non-aqueous enzymology.